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Protection from asthma in a high-risk birth cohort by attenuated P2X₇ function

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Abstract

Background—Viral illnesses are important factors in both asthma inception and exacerbations, and allergic sensitization in early life further enhances asthma risk through unclear mechanisms. Cellular damage due to infection or allergen inhalation increases ATP in the airways with subsequent purinergic receptor activation. The purinergic receptor P2X₇ may enhance airway leukocyte recruitment to the airways and P2X₇ knockout mice display a reduced asthma-like phenotype.

Objective—Based upon the P2X₇ knockout mouse, we hypothesized that children with low functioning P2X₇ would have decreased rates of asthma.

Methods—We utilized a functional assay to determine P2X₇ pore-producing capacity in whole blood samples in a birth cohort study at high risk for asthma development. The P2X₇ assay was validated with known loss-of-function alleles in humans. P2X₇ pore status categorization was used to assess asthma and allergy status in the cohort.

Results—Attenuated P2X₇ function was associated with lower asthma rates at ages 6 and 8 and the greatest effects were observed in boys. Children with asthma at age 11 who had low P2X₇ capacity had less severe disease in the previous year. Attenuated P2X₇ function was also associated with sensitization to fewer aeroallergens.

Conclusion—P2X₇ functional capacity is associated with asthma risk or disease severity and these relationships appear to be age-related.

Keywords

asthma; allergy; children; P2X₇; ATP

Introduction

Sensitization to aeroallergens and the occurrence of virus-associated wheezing illnesses are early childhood events known to increase the risk of developing asthma, and the occurrence of either is thought to be due to a balance between environmental and host factors.^{1,2} Our

group previously reported a significantly increased risk of asthma at age 6 years with acute wheezing illnesses in the first 3 years of life associated with human rhinovirus (HRV),³ and aeroallergen sensitization may contribute to the risk of more severe virus-induced wheezing illnesses and asthma.^{4–6} The risk of HRV wheeze may also depend on factors related to the virus,^{7–9} and susceptible individuals may be identified by attenuated antiviral defense mechanisms leading to compromised Type I and III interferon (IFN) production.^{10–12} Unfortunately, less is known about the control of allergic sensitization and the diverse molecular patterns and innate immune receptors comprising recognition of aeroallergens.^{13–16} Additionally, the transition from innate to adaptive immune responses is thought to be pivotal in developing sensitization.^{17–19} A recent example is the observation that chronic activation of dendritic cells (DCs) enhances development of polysensitization to new aeroallergens.²⁰ Although these findings have provided new insights, determining additional characteristics of allergen-host interactions will further identify potential interventions important in asthma.

In this regard, a growing body of evidence supports the function of nucleotides and nucleotide receptors in the regulation of innate to adaptive responses.^{15,21,22} Injury and inflammation in the lung lead to cell damage and subsequent release of intracellular danger signals in the airways including adenosine 5'-triphosphate (ATP),^{23–25} a natural ligand for a family of purinergic receptors.²⁶ Granulocytic cell influx to the airways after allergen challenge is linked to levels of ATP and is blunted when ATP is hydrolyzed or purinergic receptor antagonists are administered.^{23,27} Specifically, an absence of P2X₇ leads to a lack of pro-inflammatory cytokine interleukin (IL)-1 β release²⁸ and prevents contact dermatitis in a mouse model.²⁹ A sensitization and exposure model of allergic asthma in P2X₇ knockout mice showed decreased airway reactivity and fewer immune cells recruited to the lung after challenge.³⁰ The immunologic amplification loop involving extracellular ATP and P2X₇ has been implicated in a growing number of diseases where the resulting pathology is determined by the site at which ATP is released including at neuroreceptors, in the liver, vasculature, and the lung.^{29–33}

Since P2X₇ contributes to responses from both allergens and pathogens, we sought to assess the association between P2X₇ function and the development of asthma in a birth cohort at high risk for asthma and allergy.³⁴ The gene encoding the P2X₇ receptor (*P2RX7*) is polymorphic with non-synonymous single nucleotide polymorphisms (SNPs) resulting in functional alterations.³⁵ These functional differences allow us to utilize a flow cytometric assay to assess whether an individual's *P2RX7* genotype confers normal or loss-of-function (LOF) potential for cell membrane pore formation.^{36,37} Based upon the *P2RX7* knockout mouse,³⁰ we hypothesized that low P2X₇ pore function would confer protection from asthma. We demonstrate that low functioning P2X₇, as measured in peripheral blood monocytes, is associated with reduced risk of childhood asthma and allergic sensitization.

Methods

Study subjects

The participants were part of the Childhood Origins of Asthma (COAST) Study, a previously described longitudinal study of a birth cohort that enrolled 289 children at high risk for development of asthma.³⁴ All children had at least 1 parent with a history of physician-diagnosed asthma and/or respiratory allergies. All experiments were performed with approval of the Institutional Review Board and Human Subjects Committee at the University of Wisconsin-Madison; assent was obtained from the children, and informed consent was obtained from the children's parents.

Current asthma was defined at ages 6, 8, and 11 years as described previously³ and asthma severity was assessed at age 11 years. Briefly, current asthma was diagnosed on the basis of documented presence of 1 or more of the following in the previous year: 1) physician diagnosis of asthma; 2) use of albuterol for coughing or wheezing episodes (prescribed by a physician); 3) use of a daily controller medication; 4) step-up plan including use of albuterol or short-term use of inhaled corticosteroids during illnesses; and 5) use of prednisone for asthma exacerbation(s). Asthma severity was assessed at the 11 year visit based on National Asthma Education and Prevention Program (NAEPP) Expert Panel Report 3 (EPR-3) criteria. For children using long-term controller medications, severity was classified by the level of treatment required for control of asthma, while children not on controller therapy were classified based upon symptoms.³⁸

Wheezing respiratory tract illnesses in the first 3 years of life were previously defined by 1 or more of the following: 1) physician-diagnosed wheezing at an office visit; 2) an illness for which a child was prescribed short- or acting beta-agonists, controller medications, or both; and 3) an illness given following diagnoses: bronchiolitis, wheezing illness, reactive airway disease, asthma, and/or asthma exacerbation.³

P2X₇ pore assay

Peripheral blood samples in citrate tubes were obtained from COAST children during annual study visits at ages 10 and 11 years for whole blood pore assays to assess P2X₇ function.³⁷ Briefly, 500 μ L room temperature blood was twice with HEPES-buffered saline (HBS; 130 mM NaCl, 5 mM KCl, 20 mM HEPES, 0.1% bovine serum albumin, 10 mM glucose, pH 7.4) and incubated CD14 conjugated to phycoerythrin (CD14-PE, BD Biosciences, San Diego, HBS for 20 minutes. Samples were rinsed twice with potassium glutamate buffer (130 mM potassium glutamate, 5 mM KCl, 20 mM HEPES, 0.1% bovine serum albumin, 10 mM glucose, pH 7.4) and incubated with 250 μ M 2'(3')-O-(f-benzoylbenzoyl) adenosine-5'-triphosphate (BzATP; Sigma, St. Louis, MO) and 1 μ M YO-PRO-1 (Molecular Probes, Eugene, OR) in potassium glutamate buffer for 20 min before addition of magnesium chloride and HBS washing. Viable CD14+ cells identified by propidium iodide exclusion were examined for YO-PRO-1 median fluorescence intensity (MFI) by bead-adjusted (BD Calibrite Beads; BD Biosciences) and calibrated (RFP-30-5A; Spherotech, Lake Forest, IL) flow cytometry on a FACSCaliber (BD Biosciences). Archived DNA from COAST participants were genotyped in the lab of Dr. Carole Ober, University of Chicago. An adult population previously genotyped for *P2RX7* and with P2X₇ pore function measurements was also used for comparison.³⁷ Using our previous methods,³⁹ five functionally validated *P2RX7*LOF alleles were used to genomically validate the threshold of whole blood P2X₇ pore activity discriminating normal and attenuated function in both children and adults. A receiver operating characteristic (ROC) curve was used to instruct the threshold between low and normal P2X₇ pore activity by maximizing sensitivity and specificity in identification of *P2RX7*LOF alleles.

Allergen-specific IgE measurement

Allergen-specific IgE was measured in plasma by automated fluoroenzyme immunoassay (FEIA; Unicap 100®; Pharmacia Diagnostics AB, Uppsala, Sweden). At ages 1, 3, 6, and 9, IgE was measured for 2 species of dust mites (*Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*), *Alternaria alternata*, cat dander, and dog. At ages 6 and 9, IgE was additionally measured for ragweed, birch, timothy grass, and cockroach.⁴⁰ Tests were considered positive for values ≥ 0.35 kU_A/L.

Statistical analysis

The relationships between children's P2X₇ pore function MFI measured on different days and obtained at different ages were examined using the Pearson correlation coefficient. Logistic regression was used to examine the relationships of asthma and viral wheezing outcomes to pore status. Multivariate logistic regression models of asthma included pore status and either gender or HRV wheeze and the interaction term as covariates. The χ^2 test for association was used to compare aeroallergen sensitization rates by pore status and early life demographic and risk factors by pore status. Birth weight was compared by pore status using the Wilcoxon rank-sum test. The number of aeroallergens sensitized was compared by pore status using generalized linear mixed-effects quasi-Poisson regression models. The χ^2 test for trend in proportions was used to test the association between asthma severity and pore status. P2X₇ pore function measured by MFI of YO-PRO-1 uptake was normalized by square root transformation for analysis. A 2-sided *P* value less than 0.05 was considered statistically significant.

Results

P2X₇ Pore Assay Characteristics

At least one P2X₇ pore assay was performed on 172 children in COAST during annual visits at ages 10 and 11 years. Assay results were similar to those previously performed on adults with an approximately square root normalized distribution (Figure 1A). To validate the reproducibility of our standard methods, a subset of 48 samples had pore assays performed on more than one day post-phlebotomy with an average daily coefficient of variation (CV) of 7% between the first and second day (Pearson $r = 0.97$, $P < 0.001$; Figure 1B). Additionally, a subset of 71 children had pore assays performed at both the 10- and 11-year study visits and the year-to-year reproducibility of the assay was also highly correlated (Pearson $r = 0.91$; $P < 0.001$; Figure 1C). Overall these results confirm the high reproducibility of our pore assay and independence from potential technical confounding factors.

Because the COAST P2X₇ pore assays were consistent with previous adult assays, we combined both for ROC analysis using 5 validated *P2RX7* LOF alleles to determine a threshold between individuals with low and normal functioning P2X₇ (Figure 1D). From this analysis, a threshold of 382 MFI was identified and used to categorize all individuals with P2X₇ pore assays with either low or normal P2X₇ pore status, indicated by the shading in Figure 1A. The resulting performance properties of the assay in identifying LOF alleles for *P2RX7* are shown in Figure 1D ($P < 0.001$, area under the curve (AUC) = 0.90). Low P2X₇ pore capacity was observed in 28% of COAST participants, similar to rates in other populations.

P2X₇ pore status is independent of many demographic factors

To determine whether P2X₇ status was biased by risk factors at birth or in the first year of life, we examined the distribution of pore status across a number of risk factors for asthma. P2X₇ pore status was independent of birth and early life characteristics (Table I).

P2X₇ function, asthma inception, and disease severity

To determine the association between P2X₇ function and childhood asthma, we stratified the COAST cohort by P2X₇ pore status and determined the rates of asthma at ages 6, 8, and 11 years. Low P2X₇ pore status was associated with a decreased rate of asthma (Figure 2A) at ages 6 (Odds Ratio (OR) 0.34, 95% Confidence Interval (CI) 0.15–0.79, $P = 0.01$) and 8 (OR 0.42, 95% CI 0.20–0.88, $P = 0.02$) years, but a significant association was not observed at age 11 (OR 0.62, 95% CI 0.29–1.31, $P = 0.21$) years. To investigate whether there were

any phenotypic effects in the children with asthma at age 11 years, we also stratified the severity of asthma by P2X₇ function. Low P2X₇ pore children with asthma at age 11 years had evidence of less severe asthma compared to normal P2X₇ pore children with asthma ($P = 0.03$; Figure 2B). However, when examined at age 6 years, P2X₇ pore function was not associated with asthma severity ($P = 0.29$). Children with low pore function and asthma at age 11 years were also less likely to have used a step-up short-term plan, used for temporary loss of acceptable control with respiratory tract illnesses,⁴¹ in the previous year (OR 0.26, 95% CI 0.07–1.00, $P = 0.04$).

P2X₇ function is associated with early life wheezing with HRV

Due to P2X₇'s role in infections and airway reactivity^{30,42} and our previous observation that wheezing illnesses associated with HRV in the first 3 years of life correspond to an increased rate of asthma,³ we assessed the rates of wheezing in early life with or without virus detected based upon P2X₇ functional groups. Low P2X₇ pore status was not associated with preschool wheezing in general, but was associated with decreased wheezing associated with HRV infections in the first 3 years of life (Table II).

Decreased asthma risk associated with attenuated P2X₇ function is varied by a history of HRV wheezing and gender

To test whether reduced asthma risk in low P2X₇ individuals was only due to the association with decreased early life HRV wheezing, we modeled the interaction of P2X₇ status and HRV wheezing on asthma diagnosis by logistic regression. The greatest risk for asthma was present in children who had the combination of normal P2X₇ function and a history of HRV wheezing in the first 3 years of life (Figure 3A), and this interaction was significant at ages 6 ($P = 0.03$) and 8 ($P = 0.01$) years.

Due to different rates of asthma in boys and girls at different ages,⁴³ we also examined whether there was an interaction between gender and P2X₇ status with regard to asthma risk by logistic regression. The protective effect of low P2X₇ pore status was more pronounced in boys than girls (Figure 3B) and a significant interaction between gender and P2X₇ pore status was observed at ages 8 ($P = 0.02$) and 11 ($P = 0.03$) years. When only including boys in factoring asthma risk by P2X₇ pore status, protection from asthma in boys was significantly associated with low pore status at ages 6 (OR 0.20, 95% CI 0.06–0.64, $P = 0.004$), 8 (OR 0.19, 95% CI 0.06–0.54, $P = 0.001$), and 11 (OR 0.30, 95% CI 0.10–0.87, $P = 0.02$) years.

Allergic polysensitization and P2X₇ status

Effects of P2X₇ function on allergic sensitization rates have not previously been tested. In COAST, children with low P2X₇ function were less likely to be sensitized to common aeroallergens at age 3 years ($P = 0.04$), with a similar trend at age 9 years ($P = 0.07$; Figure 4A). Because increased activation of DCs is reported to increase rates of polysensitization,²⁰ we also examined the rates of sensitization to aeroallergens as the average number of positive sensitizations per child. When 5 common aeroallergens were modeled by mixed effects quasi-Poisson regression, low P2X₇ children were sensitized to fewer aeroallergens across ages 1, 3, 6, and 9 years (Figure 4B; mean fold change = 0.45, 95% CI 0.22–0.91, $P = 0.03$). At individual ages, low P2X₇ children were sensitized to significantly fewer allergens at age 6 years ($P = 0.02$) and low P2X₇ children trended to be sensitized to fewer allergens at ages 3 ($P = 0.09$) and 9 ($P = 0.07$) years. When including additional aeroallergens measured only at ages 6 and 9 years, a similar trend was observed, but the model no longer remained significant across both years ($P = 0.14$).

Discussion

This study adds to a growing body of research revolving around the role of nucleotides in airway disease. Similar to previous work in adults, we demonstrate good performance of a whole blood P2X₇ functional assay as a method to detect *P2RX7*LOF alleles (Figure 1). By utilizing this robust assay, we have demonstrated that a lack of P2X₇ pore activity in high-risk children is associated with a reduced risk of asthma (Figure 2), as well as sensitization to fewer aeroallergens (Figure 4). However, the mechanisms underlying these observations are not clear. Discerning the role of P2X₇ activation by extracellular ATP in concert with secondary signals including allergen exposure and/or viral infections may help determine how P2X₇ activity could modulate risk of chronic conditions such as asthma.

Previous studies indicate the amount of extracellular ATP may be related to airway disease severity.²⁵ Rather than directly measuring ATP in the airway after injurious events, our study has the strength to study the potential for differential host responses to natural *in vivo* extracellular ATP fluctuations. Our results (Figure 1D) recapitulate that considerably more contributes to P2X₇ pore function than validated *P2RX7*LOF alleles and illustrates the power of our functional approach to evaluate potential gene-by-environment interactions. The COAST population has already demonstrated gene-by-environment interactions including between *IFNG* and sex⁴⁴ which may be important to *in vivo* P2X₇ function since IFN- γ reportedly regulates P2X₇.⁴⁵ Although our current results are from pre-pubertal children, they display a varied risk of asthma by P2X₇ status based on gender (Figure 3B). Whether the dynamics of this relationship change during and after puberty will be of great interest.

Our current results are in general agreement with findings from P2X₇ knockout mice wherein low P2X₇ function is protective from asthma-like symptoms.³⁰ These P2X₇ knockout mice demonstrate decreased cell influx into the lung after allergen or smoke challenge,^{30,46} and we have previously shown a decreased neutrophil infiltration in the nose during an acute cold in adults with low P2X₇ function.⁴⁷ Although our current study may have been strengthened if P2X₇ pore assays could have been performed in early life before the earliest asthma evaluations, the high reproducibility and genetic basis of our results (Figure 1) indicates assays should be similar at any age and mitigate these potential concerns.

While low P2X₇ pore status protection from asthma in the current COAST cohort is consistent over multiple ages, these results seem counter to the inverse relationship between P2X₇ function and exacerbation risks in adults with a natural cold.⁴⁷ Differences in study populations and in the pathogenesis of asthma inception compared to exacerbations may help reconcile these findings. There are significant differences in study populations: the COAST population is comprised of high risk children followed prospectively from birth, while the previous study enrolled symptomatic asthmatic adults during the peak cold season. In the children asthma was more common in boys, while in adults asthma was predominantly observed in women. It is possible the overall lack of association at age 11 years between asthma and P2X₇ status may continue to change throughout puberty into adulthood and reflect the exacerbation risks observed in adults. Specifically, it is intriguing to note that a small percentage of children had both low P2X₇ status and a history of HRV wheezing and that this group demonstrated the largest shift in rates of current asthma at different ages. Whether modification of P2X₇ function from nucleotide activation is sufficient to alter asthma outcomes in humans has yet to be measured.

How does P2X₇ influence asthma risk? While P2X₇ is present in airway epithelial cells, the receptor is more highly expressed and active in immune cells, including DCs.^{48–50} Both

nucleotides and nucleotide receptors, including P2X₇, impact DC function^{27,30,49–52} and loss of P2X₇ function – specifically from LOF alleles detected by our pore assay – leads to a decrease in DC pore activity as well as other P2X₇-dependent functional responses.^{50,51} T cell maturation, including T_{reg} and T_H17 phenotypes, is modified by nucleotide activity upon T cells and DCs, either directly or by engaging pathways associated with P2X₇ including the NLRP3 inflammasome or pannexin-1 and suggest that functional P2X₇ activation may lead to a decrease in T_{reg} populations.^{53–57} A DC-focused role of P2X₇ is supportive of an amplified response to infections or allergens when co-mingled with danger signals acting as adjuvants. Our study demonstrates a potential role for monitoring host responsiveness to immunomodulatory danger signals.

P2X₇ sits at a balance point in the immune system in response to allergic and infectious events. It is not clear whether a single episode of P2X₇ activation is sufficient to increase the risk of asthma or whether frequent stimulation is required. Moreover, P2X₇ function may not always be beneficial or harmful in the immune response; the role of P2X₇ may be different when comparing disease inception to active, chronic conditions with superimposed acute events such as exacerbations. As examples, influenza virus activation of the inflammasome has been linked to P2X₇ function⁵⁸ while another report suggests P2X₇ may be necessary for some viruses to achieve cell entry.⁵⁹ Whether P2X₇ plays an active role in HRV infection or is secondary and solely responsive to cell injury could indicate when and where alterations of P2X₇ function are relevant. To study these relationships, the P2X₇ pore assay system described in this report is a useful tool to identify individuals at altered risk for disease and should be considered when further studying the role of danger signaling in disease pathogenesis.

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Abbreviations

ATP	adenosine-5'-triphosphate
AUC	area under the curve
BzATP	2' (3')-O-(f-Benzoylbenzoyl)adenosine-5'-triphosphate
CI	confidence interval
COAST	Childhood Origins of ASThma
CV	coefficient of variation
DC	dendritic cell
DNA	deoxyribonucleic acid
EPR-3	expert panel report 3
FEIA	fluoroenzyme immunoassay
HBS	HEPES-buffered saline

HRV	human rhinovirus
IFN	interferon
IL	interleukin
LOF	loss-of-function
MFI	median fluorescence intensity
NAEPP	national asthma education and prevention program
OR	odds ratio
ROC	receiver operating characteristic
RSV	respiratory syncytial virus
SNP	single nucleotide polymorphism

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Key Message

- P2X₇ functional capacity is easily assessed in children
- Low P2X₇ function may decrease susceptibility to diseases including asthma

Figure 1. P2X₇ pore assay characteristics

A) P2X₇ pore assay distributions for COAST (n = 172) and adult populations (n = 156) are shown. Lighter shading indicates low P2X₇ function. P2X₇ pore assays are shown for B) the same sample at different days post-phlebotomy ($r = 0.97$, $P < 0.001$) and C) at different subject ages ($r = 0.91$, $P < 0.001$). D) A ROC curve ($P < 0.001$, AUC = 0.90) is shown with characteristics for identifying P2RX7LOF alleles based on the optimized threshold of 382 MFI, indicated as the labeled point in the upper left corner maximizing both sensitivity specificity.

Figure 2. Low P2X₇ pore function is protective from asthma development

A) The rates of asthma at ages 6, 8, and 11 are shown for low and normal P2X₇ status. B) Asthma severity at age 11 is shown stratified by P2X₇ pore status. Inter = intermittent, Mod = moderate. n = number in each group. * indicates $P < 0.05$.

Figure 3. Interactions of P2X₇ with HRV wheezing and gender

Both A) P2X₇ status and HRV wheezing in the first 3 years of life and B) P2X₇ status and gender displayed an interactive effect on asthma risk. * indicates $P < 0.05$ for logistic regression model interaction terms. Lo = Low P2X₇ function, NL = Normal P2X₇ function, HRV Whz = Wheezing with HRV during first 3 years of life, M = male, F = female, n = number in each group.

Figure 4. Aeroallergen sensitization status

A) The prevalence of children with positive sensitization to any aeroallergen measured is stratified by P2X₇ function. B) The average number of allergens each subject was sensitized to was stratified by P2X₇ pore activity. For both panels, aeroallergens measured during all 4 ages are shown on the left and inclusion of additional aeroallergens measured at ages 6 and 9 is shown on the right. Error bars are the standard error of the mean. * indicates $P < 0.05$, † indicates $P < 0.1$.

Table I
P2X₇ function is independent of asthma risk factors

Subject characteristics were stratified by P2X₇ function.

Birth and year 1 risk factors	P2X ₇ pore function		P value
	Low (n = 48)	Normal (n = 124)	
Maternal asthma ever	37%	38%	0.90
Paternal asthma ever	28%	34%	0.49
Smoke exposure in year 1	27%	24%	0.69
Day care attendance in year 1	50%	50%	1.00
Exclusive breastfeeding during first 6 months	38%	35%	0.73
Dog in home at birth	40%	33%	0.42
Cat in home at birth	27%	32%	0.51
Older siblings	54%	57%	0.71
Active atopic dermatitis in year 1	30%	26%	0.59
Birth month	--	--	0.62
Birth weight $\pm$ st dev (oz)	123 $\pm$ 16	125 $\pm$ 19	0.96
Male gender	60%	56%	0.64

Table II
P2X₇ and early life wheezing

Wheezing illnesses in the first 3 years of life were compared between low and normal P2X₇ pore groups.

Wheezing illnesses in first 3 years	P2X ₇ pore function		OR [95% CI] (Low Pore)	P value
	Low (n = 48)	Normal (n = 124)		
Any cause	44%	52%	0.71 [0.36–1.38]	0.31
RSV detected	19%	31%	0.50 [0.22–1.14]	0.10
HRV detected	19%	40%	0.35 [0.16–0.79]	0.01

RSV – respiratory syncytial virus

HRV – human rhinovirus

OR – odds ratio

CI – confidence interval